AD-A283 574

UMENTATION PAGE

Form Approved
OMB No. 0704-0188

tion is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, pleting and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this ducing this burden, to Washington Headquarters Services, Directorate for information Operations and Reports, 1215 Jefferson , and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
Aug 94	

4. TITLE AND SUBTITLE
In vitro effect of sulfasalazine and its metabolites on human lymphocyte activation

5. FUNDING NUMBERS

6. AUTHOR(S)
Richard H. McBride

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
AFIT Students Attending:

Graduate College of Bowling Green State University

8. PERFORMING ORGANIZATION REPORT NUMBER
AFIT/CI/CIA
94-116

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
DEPRTMENT OF THE AIR FORCE
AFIT/CI
2950 P STREET
WRIGHT-PATTERSON AFB OH 45433-7765

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for Public Release IAW 190-1
Distribution Unlimited
MICHAEL M. BRICKER, SMSgt, USAF
Chief Administration

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

DTIC
ELECTE
AUG 19, 1994
S B D

14. SUBJECT TERMS

15. NUMBER OF PAGES
45

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
	DTIC QUALITY INSPECTED 1		

94-116

IN VITRO EFFECT OF SULFASALAZINE AND ITS METABOLITES ON HUMAN T LYMPHOCYTE ACTIVATION

Richard H. McBride

A Thesis

Submitted to the Graduate College of Bowling Green State University in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 1994

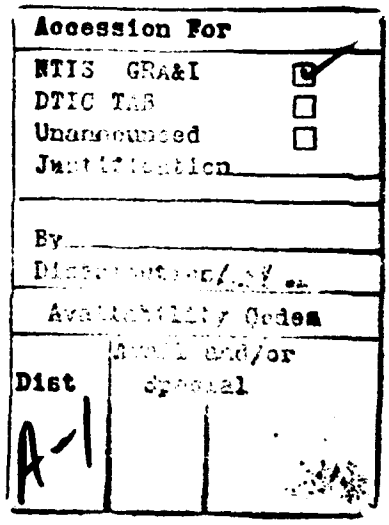
94-26438
5498
94 8 18 122

ABSTRACT

Judy Adams, Advisor

Sulfasalazine (SF) is an anti-inflammatory sulfonamide used in the treatment of rheumatoid arthritis, ulcerative colitis and ankylosing spondylitis. Its mechanism of action is not fully understood. The possible immunomodulatory role of sufasalazine and its main metabolites, sulfapyridine (SP) and 5-aminosalicylic acid (5-ASA) on T cell activation was analyzed. Peripheral blood mononuclear cells (PBMC) were separated from human blood by Ficoll-Hypaque density gradient centrifugation, depleted of monocytes by plastic adherence and further separated into B and T lymphocytes by the E-rosette method using sheep red blood cells (SRBC). Phytohemagglutinin (PHA) or anti-CD3 activated T cells were incubated in SF, SP, or 5-ASA and analyzed by flow cytometry for activation markers using fluorescein-conjugated monoclonal antibodies (FITC-mAb) or for an increase in cytoplasmic calcium ion concentration using a fluorescent calcium probe, indo-1. Sulfasalazine, at a concentration of 100 μg/mL, inhibited the expression of the interleukin-2 receptor (IL-2R, CD25), the transferrin receptor (CD71) and HLA-DR antigen in a time dependent manner. An apparent accelerative effect on the initial release of calcium from internal cellular stores of anti-CD3 activated T cells when

incubated with SF, SP or 5-ASA was not maintained throughout the 12 minute biphasic time analysis (sustained phase) of the violet/blue-green fluorescence emission ratio using indo-1. SF, and its metabolites may play a significant immunomodulatory role in T cell activation, however, more extensive studies need to be performed.



"The trouble with simple things is that one must understand them very well."

Anonymous

"Science as a culture is fundamentally chaotic,..."

Harold Varmus
Director, NIH

This work is dedicated to God, whose grace has allowed me the perseverance to see this project through to completion while granting me a vision for the future. To my mother and father for instilling in me the value of organized knowledge. To my wife, Christine, my two sons, Daniel and Jason and my daughter, Leah for their unwavering support, understanding, patience and love during an emotionally-trying time in our lives.

ACKNOWLEDGMENTS

I take this opportunity to express my appreciation to everyone who lent their support toward the successful completion of this study. First and foremost, to Dr. George Tsokos, his entire research group and the support staff in the Department of Clinical Investigation (DCI), Walter Reed Army Medical Center (WRAMC), Washington, D.C., for their invaluable assistance, advice and expertise during the carrying out of this research project. Special thanks to Lloyd C. Billups for his many hours of instruction on the use of the flow cytometer and to Capt Scott Greenwell and the entire staff of the Blood Donor Center, WRAMC, for providing fresh human blood in a timely fashion. Also, I wish to thank the Bowling Green State University thesis committee members-Drs. Judy Adams, Lee Meserve and Stan Lee Smith for their positive comments and constructive criticisms during the thesis defense. Last but not least, I wish to especially thank Dr. Dimitrios Vassilopoulos, DCI, who suggested the topic and whose constant support, advice and encouragement from proposal to defense aided tremendously in the successful completion of this study.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

STATEMENT OF PURPOSE

The mechanisms by which T lymphocytes are activated are of great importance in understanding human disease and in the development of therapeutic interventions for immune-mediated diseases[1]. One such therapy involves the sulfonamide, sulfasalazine, an anti-inflammatory drug used in the treatment of rheumatoid arthritis, ulcerative colitis, and ankylosing spondylitis[2].

The exact mode of action of sulfasalazine and its active metabolites, 5-aminosalicylic acid and sulfapyridine, as an anti-inflammatory drug, is not known[3]. Its possible role in suppression of T cells will be studied by monitoring its effect on specific early and late events in human T cell activation. The effect of all 3 compounds on the anti-CD3-induced intracellular ionized calcium concentration ($[Ca^{+2}]$) and on the phytohemagglutinin (PHA)-induced expression of cell surface activation markers in human T cells will be analyzed.

T CELL ACTIVATION

Activation of T lymphocytes is a complex process characterized by a sequence of biochemical and molecular

events (signal transduction pathway) which ultimately leads to T cell differentiation and proliferation[4] (Fig.1). Antigen recognition by T cells is the initial stimulus for T cell activation. Physiologically, the primary stimulus occurs when the T cell receptor (TCR) noncovalently binds an antigenic protein which is noncovalently bound to human leukocyte antigen (HLA) class I or II glycoproteins on the surface of an antigen presenting cell (APC)[5]. The TCR is primarily responsible for antigen recognition while the CD3 complex is primarily responsible for signal transduction. Together, antigen recognition and signal transduction via the TCR:CD3 molecular complex usually requires that: (i) the TCR recognize the HLA ligand on the surface of the APC as "self," a concept known as "MHC restriction", (ii) the APC secrete interleukin-1 (IL-1) which binds to receptors on the T cell, (iii) physical contact between the T cell and the APC occur, and (iv) accessory molecules on the surface of T cells bind to ligands on the APC[4]. Accessory molecules (CD4, CD8, CD45, gp39) contribute to the process of cellular activation by functioning as adhesion molecules, thereby stabilizing the interaction between T cell and APC; modifying the transmembrane activation signal and possibly by initiating transmembrane signal transduction distinct from the TCR:CD3 complex[1].

The TCR:CD3 complex on most T cells consists of an intrachain and interchain disulfide αβ heterodimeric

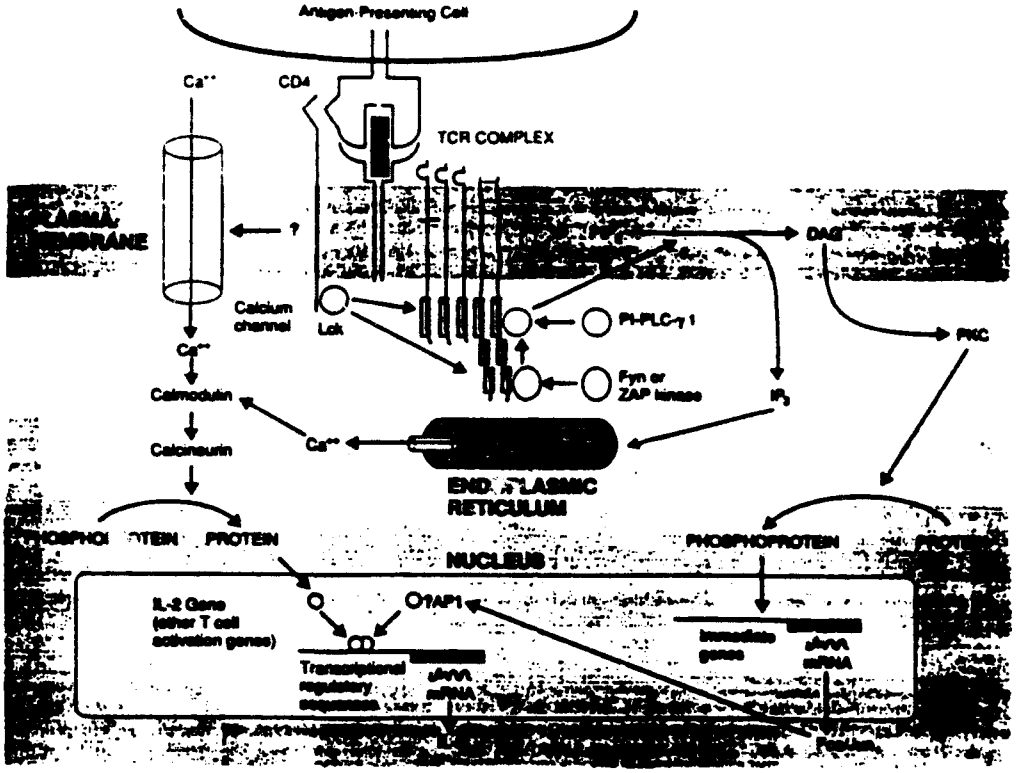


Figure 1. T cell activation leading to expression of the IL-2 gene[4]. TCR complex consists of TCR:CD3 protein chains. PIP_2=phosphatidylinositol 4,5-bisphosphate, IP_3=inositol 1,4,5-trisphosphate, DAG=diacylglycerol, PKC=protein kinase C, PLC-γ1=phospholipase C-gamma 1.

Taken from: Abbas AK, Lichtman AH, Pober JS. *Cellular and Molecular Immunology*. 2nd ed. Philadelphia: WB Saunders, 1994:159.

glycoprotein (TCR) which is physically associated with a 5 chain protein structure comprising the CD3 complex[7] (Fig.2). The CD3 protein complex consists of 3 separate monomers (γ,δ,ϵ) and either 1 homodimer ($\zeta\zeta$) or one heterodimer ($\zeta\eta$) all of which are physically associated with the TCR. The TCR:CD3 complex contains all of the information necessary for antigen and MHC specificity. It contains both constant and variable chains and is a member of the immunoglobulin gene superfamily[4].

The interaction of the TCR:CD3 complex with an HLA:antigen complex induces a transmembrane signal which is expressed via the formation of intracellular biochemical mediators called second messengers, which function as biochemical signal transmitters to stimulate transcriptional activation of a variety of genes[1]. Consequently, expression of new cell surface molecules (activation markers), cytokine secretion and mitosis leading to T cell proliferation and differentiation occur.

The events involved in T cell activation are categorized according to their relative time of occurrence[4]. Early events are defined as those which occur within seconds to hours after cell stimulation and include the biochemical production of second messengers, as well as gene transcription. Late events involve a time frame of hours to days post-stimulation and include expression of activation markers, cytokine production and mitosis. These events have

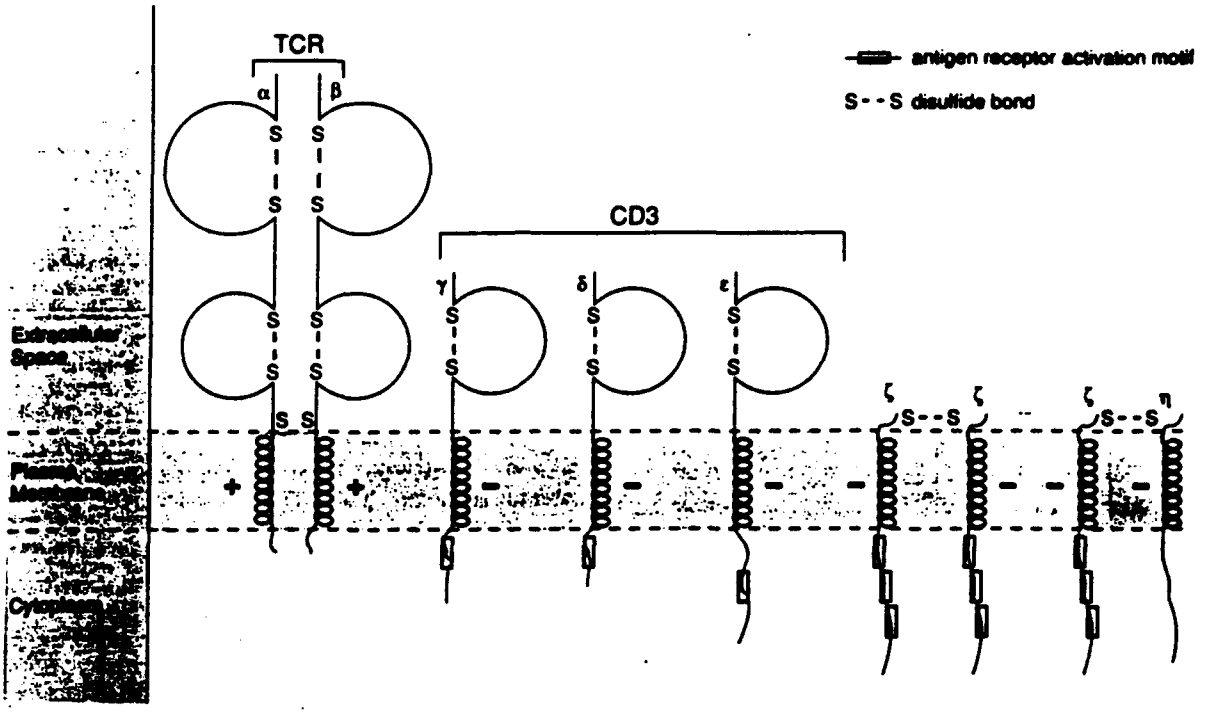


Figure 2. Components of the TCR:CD3 complex[4]. The γ,δ and ε chains of CD3 are physically associated with the TCR αβ heterodimer. Zeta (ζ) and eta (η) chains are present as ζζ homodimers or as ζη heterodimers. Immunoglobulin (Ig) homology units of 70-110 amino acid residues homologous to Ig V or C domains and containing disulfide bonds (cysteine) are present in the extracellular regions of the complex. These Ig-like domains allow the polypeptides to form a globular tertiary structure similar to antibodies. The antigen receptor activation motifs in the cytoplasmic tails of the complex are conserved sequences that include sites of tyrosine phosphorylation via protein tyrosine kinases (PTK).

Taken from: Abbas AK, Lichtman AH, Pober JS. *Cellular and Molecular Immunology*. 2nd ed. Philadelphia: WB Saunders, 1994:145.

led to the experimental analysis of various parameters to measure T cell activation such as (a) quantitation of biochemical second messengers, (b) activation marker analysis, (c) cytokine quantitation and (d) cell proliferation assays.

The main biochemical events associated with the early activation time frame are (1) phosphorylation of tyrosine residues on membrane and cytoplasmic proteins via phosphotyrosine kinases (PTK), (2) cell membrane inositol phospholipid hydrolysis, (3) increased cytoplasmic ionized calcium concentrations (4) increased protein kinase C activity and (5) gene transcription[8].

Initially, phosphorylation of protein tyrosine residues occurs within a conserved sequence of amino acid residues (antigen recognition activation motif) containing paired tyrosine and leucine groups in the cytoplasmic tails of the TCR:CD3 complex by lymphocyte-specific internal membrane src homology-2 (SH2) PTK such as lck ($p56^{lck}$, CD4), fyn ($p59^{fynT}$, CD3-ζ) and ZAP kinase (CD3-ζ)[4]. SH2 conserved domains are homologous sequences of 100 amino acids that have been associated with intracellular signal transmission pathways involving tyrosine phosphorylation[1]. As a consequence of tyrosine phosphorylation, enzymes and other proteins that also contain SH2 structurally conserved domains with tyrosine binding sites, such as the phosphodiesterase, phospholipase C-γ1 (PLC-γ1), may also bind to the tyrosine

phosphorylated membrane proteins ("docking"), resulting in enzyme activation and multiprotein complexes. Together, these activated enzymes and multiprotein complexes play significant roles in biochemical signal transmission, subsequent to TCR stimulation, during T cell activation.

Hydrolysis of membrane phospholipids is common in cells undergoing stimulation by external ligands. Within seconds of ligand:TCR binding, phosphatidylinositol 4,5-bisphosphate (PIP_2) is hydrolyzed to diacylglycerol (DAG) and inositol 1,4,5 trisphosphate (IP_3) in a PLC-γ1 catalyzed reaction. The two second messenger products, DAG and IP_3, trigger two parallel pathways that act in concert to trigger the same cellular response.

IP_3 stimulates the rapid release of intracellular calcium stores from the endoplasmic reticulum within seconds of activation via a specific receptor. The rapid and sustained increase in intracellular ionized calcium (Ca^{+2}) is maintained for over an hour, implying dependence on a transmembrane flux of extracellular calcium, probably through a calcium ionophore response to phospholipid hydrolysis[1]. The calcium binds to calmodulin, a calcium sensor which serves as a regulatory protein of intracellular enzymes and biochemical signal receiver in all eukaryotic cells[9]. The binding of calcium to 4 sites in calmodulin induces the formation of an α-helix and other conformational changes that convert this 17 kD protein to an active state.

Activated calmodulin either stimulates or inhibits bound intracellular enzymes. In T cells, calcium:calmodulin complexes primarily activate calcineurin, a serine and threonine protein phosphatase that is believed to play a role in the primary expression of the IL-2 gene[1].

The other second messenger product of membrane phospholipid hydrolysis, DAG, activates protein kinase C, a serine and threonine protein phosphokinase believed to be responsible for the transcriptional activation of specific cellular proto-oncogenes (c-fos/jun, AP-1) necessary for the transcriptional activation of the IL-2 gene[4]. Phosphorylation and dephosphorylation of serine, threonine and tyrosine residues on accessory molecules, enzymes, DNA-binding proteins, transcriptional factors and other regulatory proteins present in the cytoplasm and catalyzed by protein kinase C, phosphatases (calcineurin, CD45) and other kinases play a significant role in the transcriptional activation of several genes essential for T cell activation.

After thymic ontogeny, T cells migrate through the peripheral blood and circulate until activated by an antigen:HLA complex. Upon activation, a 7-10 day process begins which results in cell proliferation and differentiation. The activated cells undergo blastogenesis (12 hours), mitotic division (24-72 hours) and differentiate as genes are sequentially activated[10]. These physiologic events can be mimicked *in vitro* through the use of calcium

ionophores, lectins, monoclonal antibodies and phorbol esters[4,9,10]. Ionophores, such as ionomycin, are calcium-specific proteins with lipophilic moieties which allow them to traverse the cell membrane, transporting calcium to the cytosol. Lectins, which bind to specific carbohydrate residues on membrane bound glycoproteins, include phytohemagglutinin (PHA), derived from kidney beans, and concanavalin-A (Con-A), derived from jack beans. Phorbol esters such as phorbol myristate acetate (PMA), are naturally occurring, as well as, synthetic tumor promoters which are chemically homologous to DAG and therefore, capable of activating protein kinase C. Phorbol esters and calcium ionophores act synergistically to promote T cell activation, however, neither can sustain T cell activation alone. All the aforementioned agents are collectively known as polyclonal activators since they have the ability to bind to T cells, regardless of antigenic specificity and mimic physiologic stimulation of the TCR:CD3 complex[4].

Over 70 genes are activated at times ranging from 15 minutes to 14 days during T cell activation and categorized as immediate, early or late on the basis of time required for earliest expression of messenger RNA (mRNA)[(10)]. Genes in the first two categories (immediate, early) are transcribed prior to mitosis (24-72 hours post activation) and include c-fos, c-myc and IL-2, while genes in the third category (late) are transcribed after mitosis.

The interleukin-2 receptor (IL-2R, CD25) and the transferrin receptor (CD71) are both considered products of early activated genes based on their earliest mRNA detection at 2 hours and 14 hours, respectively[11,12]. The transcription of the IL-2R gene is necessary for the auto-stimulation of the same T cell in response to IL-2 secretion, as well as, stimulation of other T cells. The autocrine and paracrine function of IL-2 requires the formation of a tetramolecular complex consisting of IL-2 and three distinct cell surface proteins which are 55 kD, 70-75 kD and 64 kD, respectively, comprising the interleukin-2 receptor. Only activated T cells express the 55 kD peptide (p55), known as IL-2Rα. It binds IL-2 with an equilibrium dissociation constant (K_d) of ~10^{-8}M[4], however, binding of IL-2Rα alone with IL-2 elicits no biologic response.

The 70-75 kD peptide, IL-2Rβ, is a member of the WSXWS sequence motif cytokine receptor family[4]. It contains a conserved extracellular sequence of 5 amino acids, tryptophan-serine-X-tryptophan-serine, where X represents any amino acid. This conserved sequence homology is present in several cytokine receptors and is usually located just outside the transmembrane region. The K_d of IL-2Rβ is ~10^{-9}M, indicating a higher binding affinity than IL-2Rα for IL-2. IL-2Rβ is physically associated with IL-2Rγ, another WSXWS sequence homology protein (64 kD). Resting (G_0) cells express only IL-2Rβγ. Cells that express high concentrations

of both IL-2Rα and IL-2Rβγ (activated T cells) have the highest binding affinity, with a K_d of approximately 10^{-11}M. The ability of activated T cells to secrete IL-2 is directly proportional to the concentration of the IL-2R protein complex, allowing for a positive feedback amplification system. In other words, initial binding of the IL-2 cytokine receptor on the cell membrane surface with IL-2 leads to the formation of a relatively higher affinity IL-2 receptor that binds IL-2 more intensely, thereby stimulating T cell proliferation[12].

The transferrin receptor (CD71) is a 100 kD nutrient glycoprotein expressed on the surface of activated T cells. The binding of serum transferrin to cell surface receptors is required for cell growth and precedes the initiation of DNA synthesis[13].

Class II human leukocyte antigen markers (HLA-DR) are expressed on activated T cells beginning 3-5 days post activation and are therefore considered products of late activated major histocompatibility complex (MHC) genes[14]. T cell activation results in greater than a tenfold increase in the membrane expression of HLA-DR proteins. The transferrin receptor and the IL-2R exhibit an average fivefold and fiftyfold increase in expression, respectively, as a consequence of T cell activation[10].

SULFASALAZINE

Sulfasalazine (SF) is a broad spectrum sulfonamide which consists of sulfapyridine (SP) linked to 5-aminosalicylic acid (5-ASA) by a diazobond (Fig.3). After oral ingestion, the drug passes unchanged through the small intestine and is reduced by colonic bacterial azoreductases[2].

In 1987, Comer and Jasin[15] studied the *in vitro* effect of sulfasalazine, sulfapyridine and 5-aminosalicylic acid on peripheral blood lymphocyte function of normal and rheumatoid arthritis (RA) patients. Sulfasalazine inhibited mitogen (pokeweed, PHA, Con-A) induced mononuclear cell proliferation at a concentration of 100 μg/mL. Sulfasalazine also depressed mitogen induced immunoglobulin synthesis in normal and RA patients. Also, synthesis of rheumatoid factor was supressed more than total IgM at sulfasalazine concentrations of 10-25 ug/mL. Neither SP nor 5-ASA exhibited inhibitory function.

Sheldon, Webb and Grindulis[3], in 1987, examined the *in vitro* effect of sulfasalazine and its two main metabolites on the mitogen induced lymphocyte transformation of murine splenocytes. A consistent and profound suppressive effect was noted with sulfasalazine in doses ranging from

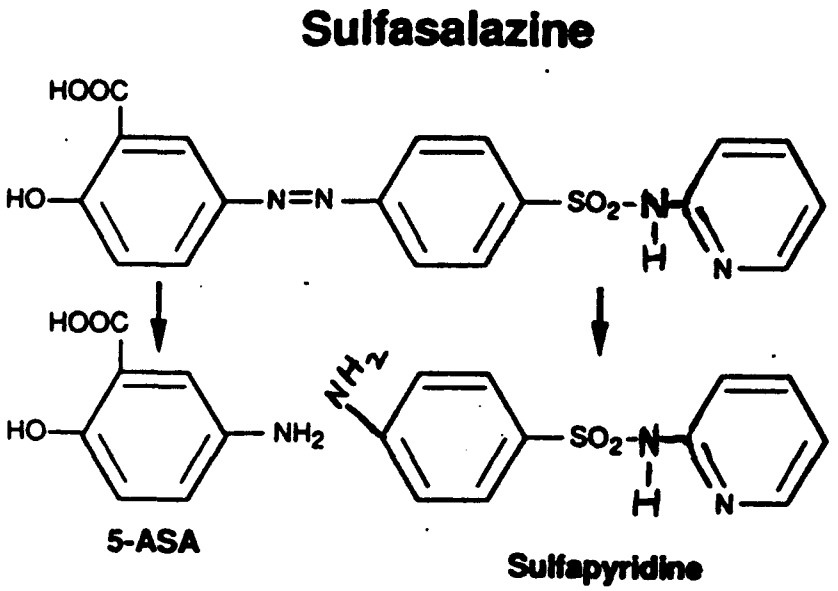


Figure 3. Chemical structure of sulfasalazine, sulfapyridine and 5-aminosalicylic acid (5-ASA)[2]. Seventy to eighty percent of SF passes unchanged through the small intestine into the colon, where it is reduced by bacterial azoreductases into its two main metabolites, SP and 5-ASA.

Taken from: Allgayer H. Sulfasalazine and 5-ASA compounds. *Gastroenterol Clin North AM* 1992;21:645.

25-100 μg/mL. No suppression was noted with sulfapyridine and 5-aminosalicylic acid. A concentration of at least 50 ug/mL sulfasalazine was needed to obtain >50% suppression and was higher than the concentration ever found in the blood of humans on a prescription drug regimen.

In 1991, Samanta, et al[16] analyzed lymphocytes from rheumatoid arthritis patients receiving sulfasalazine to determine qualitative and quantitative differences. After 12 weeks of therapy, no change in the absolute number or percentage of peripheral blood lymphocytes expressing CD3, CD4, CD8, CD24 or HLA-DR antigens was noted. Qualitatively, patient's lymphocytes after 12 weeks of therapy showed no statistically significant suppression after PHA induced activation at 0 and 12 weeks.

Neutrophils play a significant role in rheumatoid arthritis and other inflammatory conditions. Upon activation, neutrophils produce reactive superoxide radicals which provoke tissue destruction and mediate abnormal immune responses. In 1989, Kanerud, Hafstrom and Ringertz[17] investigated the effect of sulfasalazine and sulfapyridine on neutrophil superoxide anion generation. Superoxide production in neutrophils was induced via the calcium ionophore A23187, the synthetic protein N-formyl-methionyl-leucyl-phenylalanine (fMLP) and the phorbol ester, phorbol myristate acetate (PMA). Neutrophils were isolated by Ficoll Hypaque density gradient centrifugation and incubated in

culture medium at 1 μmol/L for 10 minutes. Superoxide concentrations were measured spectrophotometrically. Cytosolic free calcium was measured with a fluorescent chelating probe (fura-2) because of its bright fluorescence and selectivity toward calcium. The neutrophils were buffered with 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) and fluorescence measured with a spectrophotometer. The authors stated that both SF and SP suppressed neutrophilic superoxide anion production when induced by both fMLP and A23187. The effect appeared to be dependent on inhibition of intracellular calcium increases. No suppression effect was noted with PMA activated neutrophils.

In 1991, Sheldon, Pell and McBurney[18] reported that sulfasalazine appeared to exert a mild immunosuppressive effect on murine mucosal immune systems. Mice administered cholera toxin and sulfasalazine simultaneously over a one month period were analyzed for circulating antibodies using ELISA techniques at 1 week intervals. Mice receiving SF tended to produce lower levels of antibodies to cholera toxin.

In summary, sulfasalazine appears to exhibit a mild immunosuppressive effect on T and B lymphocytes, as well as, neutrophils. No such effect was noted with the metabolites, SP and 5-ASA on lymphocytes, however, SP does appear to inhibit neutrophil activation *in vitro*.

CALCIUM

As previously stated, ligand-initiated cell stimulation in T cells leads to activation of the phosphatidylinositol (PIP_2) second messenger pathway and results in a rapid increase in cytoplasmic, free, ionized calcium (Ca^{+2}). Calcium, a common biochemical second messenger, is present in resting T cells at ~10^{-7}M. Upon T cell activation, calcium ionophores allow a sustained influx of extracellular calcium (~10^{-3}M), making it an excellent indicator of cellular changes. The development of calcium-sensitive, fluorescent chelating dyes (indo-1, quin-2 and fura-2) has lead to the ability to quantitate the cytoplasmic calcium concentration ([Ca^{+2}]) and therefore, determine the extent of cell activation via the second messenger pathway using flow cytometry[19]. Indo-1 is introduced into the cell (loading) as a lipophilic acetoxymethyl (AM) ester that is permeable to cell membranes (Fig.3). Once inside the cytosol, non-specific esterases hydrolyze the calcium-binding ester to acetic acid and formaldehyde, releasing the dye as an intracellular, anionic chelator, trapped by the electrically charged plasma membrane, which carries a resting negative electrical potential of ~70 millivolts(mV). Intracellularly, the fluorescent dye binds Ca^{+2} in a reversible equilibrium and fluoresces in proportion to the calcium concentration[19]:

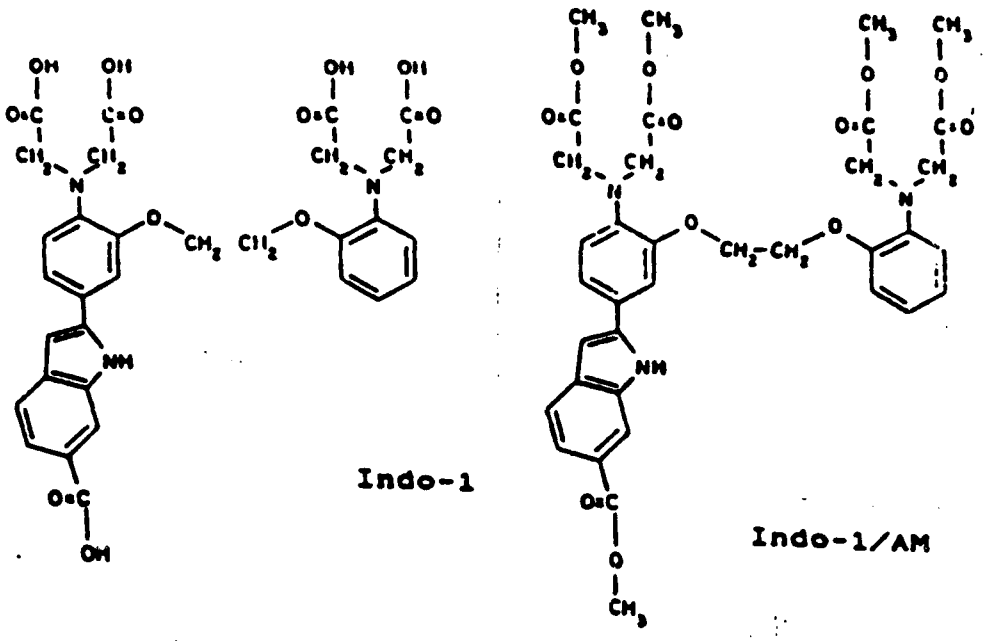


Figure 4. Chemical structure of indo-1 acid and indo-1 acetoxymethyl ester (AM). The lipophilic ester traverses the phospholipid cell membrane. Intracellular esterases hydrolyze the methyl groups, resulting in an anion that is membrane impermeant and capable of chelating calcium.

Taken from: *Indo-1 and Calcium in Flow Cytometry*, Indo-1 Workshop, Florida: Coulter Corporation, 1990:7.

$$[Ca^{+2}] + [\text{Indo-1}]_{free} = [Ca^{+2}/\text{Indo-1}] \qquad (1)$$

Indo-1 AM ester, a fluorescent calcium probe, which requires an electromagnetic radiation source that emits in the UV region, such as an argon laser (351.1, 363.8 nm) or a helium-cadmium laser (325 nm), allows a ratio analysis of calcium. This is possible because free indo-1 fluoresces at ~500 nm (blue-green), but shifts to ~400 nm (violet) when bound to calcium (Fig.4). The violet/blue-green fluorescence emission ratio is proportional to the ratio of [bound indo-1]/[free indo-1], which is proportional to the concentration of intracellular calcium ($[Ca^{+2}]$), (Eq.1). The measurement of intracellular ionized calcium using indo-1 was first described by Rabinovitch, June, Grossmann and Ledbetter in 1986[20]. Their experiments showed that the intracellular ionized calcium response to external agonists could be effectively analyzed by flow cytometry using indo-1. They demonstrated the heterogeneous calcium response of different subpopulations of T cells to externally applied mitogenic activators such as anti-CD3 antibody or PHA. The calcium response was greater among CD4+ cells compared to CD8+ cells. Within the CD4+ subset of T cells, those expressing the p220 antigen exhibited a slower calcium response. These results reinforced the suggestion that CD3-mediated calcium signals are transduced differently in various subpopulations of T cells. Rabinovitch *et al*

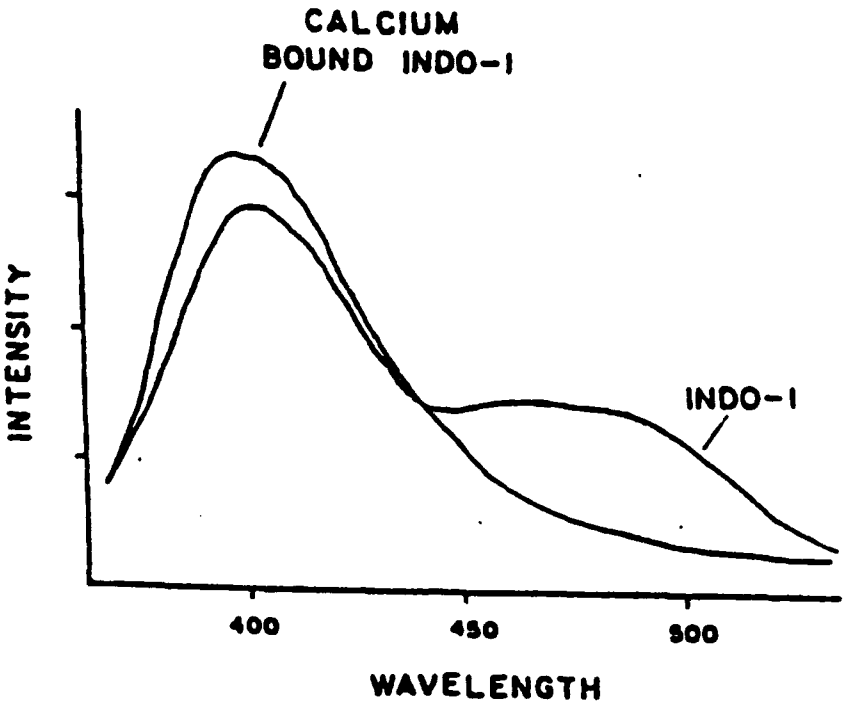


Figure 5. Indo-1 fluorescence emission spectrum. Free indo-1 fluoresces at ~500 nm (blue-green), while bound indo-1 fluoresces at ~400 nm (violet). This spectral property allows a ratio analysis of the intensity of violet/blue-green fluorescence which is proportional to the intracellular calcium concentration ($[Ca^{+2}]$).

Taken from: *Indo-1 and Calcium in Flow Cytometry*, Indo-1 Workshop, Florida: Coulter Corporation, 1990:10.

measured a maximum sixfold increase in the magnitude of the violet/blue-green fluorescence emission ratio when they measured intracellular calcium levels in resting and activated indo-1 stained T cells. They stated the significance of emission ratios which eliminated dependence on variations in loading concentrations of indo-1.

In 1986, June, Ledbetter, Rabinovitch, *et al*[21] evaluated anti-CD2 and anti-CD3 triggered activation of resting (G_0) lymphocytes by measuring intracellular free calcium concentrations of individual cells, using indo-1 ratios measured by flow cytometry. Stimulation with anti-CD3 antibody caused an increase in intracellular calcium in >90% of CD3+ cells within one minute. The response was limited to CD3+ cells. Stimulation of cells with anti-CD2 antibodies produced a biphasic calcium response pattern, with an initial peak in CD3- cells and a sustained peak in CD3+ cells (6 minute lag phase). The CD2-mediated response did not require the presence of CD3 on the surface membrane of the cells. The anti-CD2 mAb-mediated response of CD3- cells showed no lag phase. They concluded that the changes in cytosolic calcium could have resulted from intracellular mobilization (calcium stores) or extracellular transport (calcium ionophores). Quantitatively, most of the calcium increase after CD2 or CD3 stimulation of G_0 T cells appeared to be the result of transport of Ca^{+2} from the extracellular space. Their findings supported the hypothesis that

engagement of the CD2 surface molecule initiates an alternate pathway to T cell activation.

Finkel, McDuffie, Kappler, *et al*[22] in 1987, investigated the proposed differences in signal transduction following T cell activation via the TCR:CD3 complex in immature and mature T cells. Intracellular calcium mobilization was measured in both sets of lymphocytes. Their findings suggested that antigen receptors on both mature and immature receptor- positive T cells were capable of transducing signals via calcium mobilization. Immature cells expressed a reduced calcium influx response compared to mature cells. Release of calcium from intracellular stores was similar in both mature and immature cells. Extracellular influx of calcium was markedly reduced in immature cells. These findings may imply a weaker calcium channel in immature T cells or inefficient activation of existing channels in immature cells.

June, Rabinovitch and Ledbetter[23], in 1987, demonstrated that monoclonal antibodies to CD5 caused an increase in intracellular free calcium concentrations in T cells. The increase was detectable 1 minute after addition of CD5 antibodies in indo-1 loaded cells. Calcium increases induced by suboptimal CD3 antibodies were supplemented and sustained by CD5 antibodies. The CD5 calcium response was expressed by CD5/CD3 positive cells only. CD5 induced calcium mobilization was inhibited by PMA whereas the CD3

response was not. This suggests that protein kinase C-depleted CD5 T cells caused an uncoupling of signal transduction between CD5 and calcium channels. These findings were consistent with the suggestion that one mechanism for CD5-induced supplementation of mitogen stimulated T cell proliferation involved increased cytosolic calcium, which was distinct from, but dependent on, stimulation of CD3.

In 1988, Gelfand, Cheung, Mills and Grinstein[24] measured intracellular calcium increases as a function of time in mitogen-induced human T lymphocytes loaded with indo-1. A biphasic fluorescence time curve consisting of a peak ratio produced by the initial calcium release from intracellular stores and a plateau region which reflected a calcium influx (sustained phase) from the extramembranous region through a calcium ionophore, was recorded. A high affinity intracellular calcium chelator (BAPTA) which neither contributes to nor interferes with fluorescent determinations was used to assess the relative effects of extracellular calcium on initial and sustained phases. In cells loaded with BAPTA, T cell stimulation with mitogen or monoclonal antibodies failed to elicit the initial peak. Only the plateau region (sustained phase) was observed fluorimetrically in the presence of extracellular calcium. BAPTA-loaded cells expressed IL-2R, secreted IL-2 and underwent mitosis. Transient increases in intracellular

calcium from the release of internal stores did not appear to be essential for T cell activation, but sustained influx of extracellular calcium via specific ionophores seemed to be required.

Goldsmith and Weiss[25], in 1988, measured inositolphospholipid second messengers and early calcium mobilization in parental leukemic and somatic mutant T cell lines with deficient receptor function. Several monoclonal antibodies were directed against the receptor complex and measured for their ability to elicit transmembrane signaling. One antibody elicited calcium mobilization in both cell lines, but was unable to induce transcriptional activation of the IL-2 gene. In the somatic mutant T cell line, there was diminished production of phospholipid second messengers and increases in cytosolic calcium concentrations were not sustained. Their interpretation of the results was that a calcium influx must be sustained longer than 2 hours to promote IL-2 gene expression and that an initial (transient) rise is not sufficient for total signal transmission leading to cell activation. A sustained second messenger generation was required for transcriptional activation of the IL-2 gene.

In summary, the accurate measurement of intracellular calcium increases following cell activation via synthetic phorbol esters, ionophores or monoclonal antibodies has been successfully demonstrated using indo-1 and flow cytometry.

Specific fluorescent chelators have reliably reproduced both the transient calcium response (initial peak) from stored intracellular compartments, as well as, the influx of extracellular calcium via membrane channels. In addition, engagement of different T cell surface markers (CD2, CD3, CD5) is characterized by distinct signaling processes. Although all of them result in early peak and plateau Ca^{+2} responses, prolonged increase in $[Ca^{+2}]$ is necessary for successful T cell activation.

MATERIALS AND METHODS

ACTIVATION MARKERS

LYMPHOCYTE SEPARATION, ACTIVATION AND CULTURE. Fresh, citrated blood from volunteer donors was provided by the Blood Donor Center, Walter Reed Army Medical Center, Washington, D.C. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation[26] using Histopaque-1077 (Sigma, St. Louis, Missouri) and resuspended at a concentration of 5 x 10^6 cells/mL in RPMI-1640 (Gibco, Frederick, Maryland) with 20% fetal calf serum (FCS, Sigma). All RPMI medium was supplemented with 12.5 mL of 1M HEPES buffer, 10 mL of 0.2M L-glutamine, 5 mL of 10,000 U/mL penicillin and 10,000 μg/mL streptomycin (Gibco, Frederick, Maryland). PBMC were depleted of monocytes by plastic adherence according to the method of Djeu[6]. Cells were incubated at 37C in horizontally positioned, loosely capped polystyrene culture flasks in RPMI-10 for 2 hours in a 5% CO_2, humidified incubator. Afterwards, cells were rinsed in RPMI-10 and the resulting non-adherent cell suspension (lymphocytes) was maintained at 5 x 10^6 cells/mL in RPMI with 20% FCS. Further separation of T lymphocytes from B lymphocytes was obtained using the lymphocyte E(erythrocyte)-rosette method[28] with sterile, defibrinated sheep red blood cells (SRBC, Waltz Farm,

Smithsburg, Maryland). Cells were incubated with 10% SRBC overnight, underlayed with Ficoll-Hypaque solution, centrifuged at 1400 rpm (Jouan, model #C4-12) for 40 min and the supernatant discarded. Resulting SRBC-T cell precipitate was separated using ammonium chloride-potassium bicarbonate (NH_4Cl-$KHCO_3$) red blood cell lysing solution to obtain a final concentration of 2 x 10^6 T cells/mL in RPMI with 10% FCS (RPMI-10). To determine cell concentrations and viability, cells were diluted 1:20 (1:19 v/v) in 0.4% trypan blue (Sigma) and counted with a Neubauer hemacytometer using the trypan blue dye exclusion technique[28].

One mL aliquots (2 x10^6 T cells) were simultaneously activated and cultured in 24 well, flat bottom, polystyrene plates with 0.5 mL of 4 μg/mL PHA solution and 0.5 mL of 400 μg/mL SF, SP or 5-ASA solutions. Final concentration of PHA and any drug in all working wells was 1 μg/mL and 100 μg/mL, respectively. The 4 μg/mL PHA working solution was prepared by using RPMI-10 to make a 1:250 dilution of a 1 mg/mL PHA stock solution prepared by dissolving 2 mg of powdered PHA (Murex Diagnostics, Norcross, Georgia) in 2 mL of phosphate buffered saline (PBS). PHA stock solution was stored at -20C. The 400 μg/mL working solutions of SF (Sigma) and SP (Sigma) were prepared by making 1:100 dilutions with RPMI-10 of a 40 mg/mL stock solution made by dissolving 2 grams of SF or SP in 5 mL of heated 0.5M NaOH. The stock solutions of SF and SP were brought to 50 mL with

distilled water and stored at 4C. The 400 μg/mL working solutions of 5-ASA (Sigma) were made fresh (to avoid unfavorable oxidation reactions when stored) by diluting 1:50 with RPMI-10 from a 20 mg/mL stock solution which had been prepared by dissolving 0.05 grams in 0.4 mL of 1M HCl, then brought to 2.5 mL with distilled water. Control wells consisted of cell solutions with medium alone and cell solutions incubated in SF, SP or 5-ASA without PHA. In addition, cells in PHA were incubated in a control medium consisting of aliquots from pH stock solutions which approximated the pH of SF, SP and 5-ASA stock solutions (11.5, 13.2 and 1.5, respectively). The pH-specific controls were diluted appropriately (either 1:100 or 1:50) in RPMI-10 with no drug added (0 μg/mL). Total final volume in all working wells before incubation was 2 mL. All cell solutions were incubated for 1, 3 or 5 days in a carbon dioxide enriched (5%), humidified, 37C incubator.

IMMUNOSTAINING AND FLOW CYTOMETRY. After 1, 3 or 5 days, cell cultures were visually inspected using light microscopy (100X) for signs of cell death or activation. Five aliquots (0.35 mL) of cell solution from each well were placed in each of 5 polystyrene test tubes and washed twice with cold, filtered PBS supplemented with 1% FCS and 0.01% sodium azide (NaN_3). For each wash, the tubes were centrifuged at 1300 rpm (DuPont Sorvall, model #RT6000D) for 5 min at 4C

followed by decantation of supernatant, leaving 200-300 μL of solution in test tubes. Cells in each test tube were then immunostained with 5 μL of one of five different monoclonal antibodies (mAb) conjugated with fluorescein isothiocyanate (FITC): anti-CD25, anti-CD71 or anti-HLA-DR, as well as, IgG2a (CD25, HLA-DR) or IgM (CD71) isotypic controls (Coulter). Test tubes were incubated for 35 min in the dark at 4C. After cold incubation with mAb, cells were again washed twice with cold PBS/1.0% FCS/0.01% NaN_3 and the solution decanted, leaving 200-300 μL in each test tube. Cells were cytopreserved in 1% paraformaldehyde. Flow cytometric analyses were performed on the Epics Elite flow cytometer (Coulter). Excitation was provided by an argon laser (488 nm, 15 mW). Activation marker expression was quantified by analyzing 5,000 cells on the cytometer using the histogram obtained with the isotypic control for the mAb as the negative control. Fluorescence intensity histograms and dual parameter dot plots were analyzed with the Epics Elite computer software package (Coulter) to calculate the mean difference in percent of cells expressing IL-2R, CD71 or HLA-DR antigen at stated concentrations of SF and its two metabolites. Percent inhibition of activation marker at a drug concentration of 100 μg/mL compared to control (0 μg/mL) was calculated using the formula:

$$\text{PERCENT INHIBITION} = [1-(X_{100}/X_0)]*100 \qquad (2)$$

where X represents the mean percent of cells at a drug concentration of 100 μg/mL or 0 μg/mL, expressing an activation marker at day 1,3 or 5 of culturing in the presence of the drug. No more than one unit of blood, representing one donor, was used for a complete (5 days or less) experiment at a time.

STATISTICAL ANALYSIS. The Student's independent *t* test was used to analyze the mean difference and standard error of the mean (SEM) in percent of T cells expressing activation markers at stated concentrations of SF, SP and 5-ASA. Significance was defined as $p<0.05$.

MEASUREMENT OF FREE INTRACELLULAR CALCIUM

T cells were isolated as described above and suspended at 5 x 10^6 cells/mL in RPMI-10. One mL of cells was incubated overnight in 0.5 mL of SF, SP, or 5-ASA (400 μg/mL) and 0.5 mL of RPMI-10 medium for a final concentration of 100 μg/mL of each drug in a total volume of 2 mL. One mL of the cell suspension in 1 mL of RPMI-10 served as a control. All samples were cultured in 24-well flat bottom polystyrene plates at 37C in a 5% CO_2, humidified incubator. After incubation, each cell culture sample was washed once to remove the drug. Each sample volume (2 mL) was transferred to a 15 mL centrifuge tube,

filled with RPMI, centrifuged at 1300 rpm (Jouan, model #C4-12) for 5 min at room temperature, then resuspended in 1 mL of fresh medium. Fifty μg of Indo-1 AM (Molecular Probes, Eugene, Oregon) was dissolved in 50 μL of dimethyl sulfoxide (DMSO, Sigma) for a final concentration of 1 μg/uL, separated into 10 μL aliquots and stored frozen (-70C) until use. When needed, 10 μL aliquots of indo-1 AM (8 μM) were mixed in 200 μL of RPMI with 1% fetal calf serum (RPMI-1), added to the 1 mL cell culture samples and incubated in a 37C heat rack in the dark for 30 min with periodic shaking. After incubation, three 200 μL aliquots of each sample were prepared. Two of the aliquots were immunostained for 20 minutes in the dark with 5 μL of a fluorescent conjugated mAb to either CD3 (anti-CD3-FITC, Coulter) or to B cells (anti-CD19-phycoerythrin (PE), Coulter) and monocytes (anti-CD11b-FITC, Coulter). The third aliquot was left unstained. Afterwards, cells were washed with RPMI-1 to remove unbound mAb and centrifuged at 1000 rpm (Beckman, model #GS-6R) for 5 min at room temperature. After centrifugation, the cell supernatant was decanted or pipetted until 200-300 uL of cell solution were remaining. A 100 μL cell sample from each tube was diluted in 400 μL of prewarmed (37C) RPMI-1 supplemented with 1mM of $CaCl_2$ (1:5 dilution), activated with 10 μL of a 1 mg/mL potent murine monoclonal anti-CD3 antibody (G19-4, Bristol-Myers Squibb, Seattle, Washington) and analyzed with a flow cytometer

(Coulter) equipped with both an argon (488 nm, 15 mW) and an air cooled, helium-cadmium, ultraviolet (325 nm) laser. T cells were negatively selected for analysis by gating out both B cells and monocytes using anti-CD19-PE and anti-CD11b-FITC, respectively. The T cell activator (anti-CD3, G19-4) was added to the sample 1 min after initiation of analysis to establish a baseline (G_0 calcium level) emission ratio. The mean violet/blue-green emission ratio of 381 nm (indo-1 with calcium) to 525 nm (free indo-1) was recorded as a function of time over a period of 12 min and analyzed to determine differences in calcium ion responses among the samples. Bound indo-1 emission measurements were facilitated by the use of a 440 nm long pass dichroic mirror coupled to a 381 nm bandpass filter to transmit reflected radiation to a UV short (381 nm) photomultiplier tube (PMT). Free indo-1 emission measurements were facilitated by the use of a 550 nm long pass dichroic mirror coupled to a 525 nm bandpass filter in order to transmit reflected radiation to the UV long/FITC (520-530 nm) PMT. To eliminate simultaneous detection of fluorescence at ~525 nm emitted by both FITC (CD11b, monocytes) and free indo-1, a gated amplifier was used to delay absorption (~60 μsec) of the 488 nm argon light by anti-CD11b FITC. Data were analyzed via the Multitime computer program (Phoenix Flow Systems, San Diego, California).

RESULTS

EFFECT OF SF AND ITS METABOLITES ON ACTIVATION MARKERS. The *in vitro* effect of sulfasalazine (SF) and its two main metabolites, sulfapyridine (SP) and 5-aminosalicylic acid (5-ASA), on the PHA-induced expression of CD25 (IL-2R), CD71 (transferrin receptor) and HLA-DR antigen and on the anti-CD3 induced mobilization of calcium was investigated (Fig. 6-9). The decrease in expression (percent inhibition) of the IL-2 receptor at an SF concentration of 100 μg/mL compared to 0 μg/mL, varied from 39% at day 1 to 0% by day 5 of drug incubation (Fig.6). The 39% represented the difference in the mean percent of cells ± the standard error of the mean (SEM) expressing the IL-2R at the two SF concentrations for N experiments (40.5 ± 8.9%, SF=0 μg/mL vs. 24.6 ± 19.2%, SF=100 μg/mL, N=5, $p>0.05$). Specimens cultured in SP exhibited a measurable decrease in IL-2R expression (~10%) at day 5, however, the result represented only 2 experiments (N=2). The percent inhibition of the IL-2R in 5-ASA remained relatively constant throughout the time course (5 days) of the cultures.

The percent inhibition of CD71 in SF ranged from a high of 79% on day 1 of culture (16.9 ±3.2%(SEM) of cells expressed CD71 at 0 μg/mL SF vs. 3.6 ± 1.0%(SEM) of cells at 100 μg/mL SF, N=5) to 15% on day 5 after culture (N=2). The

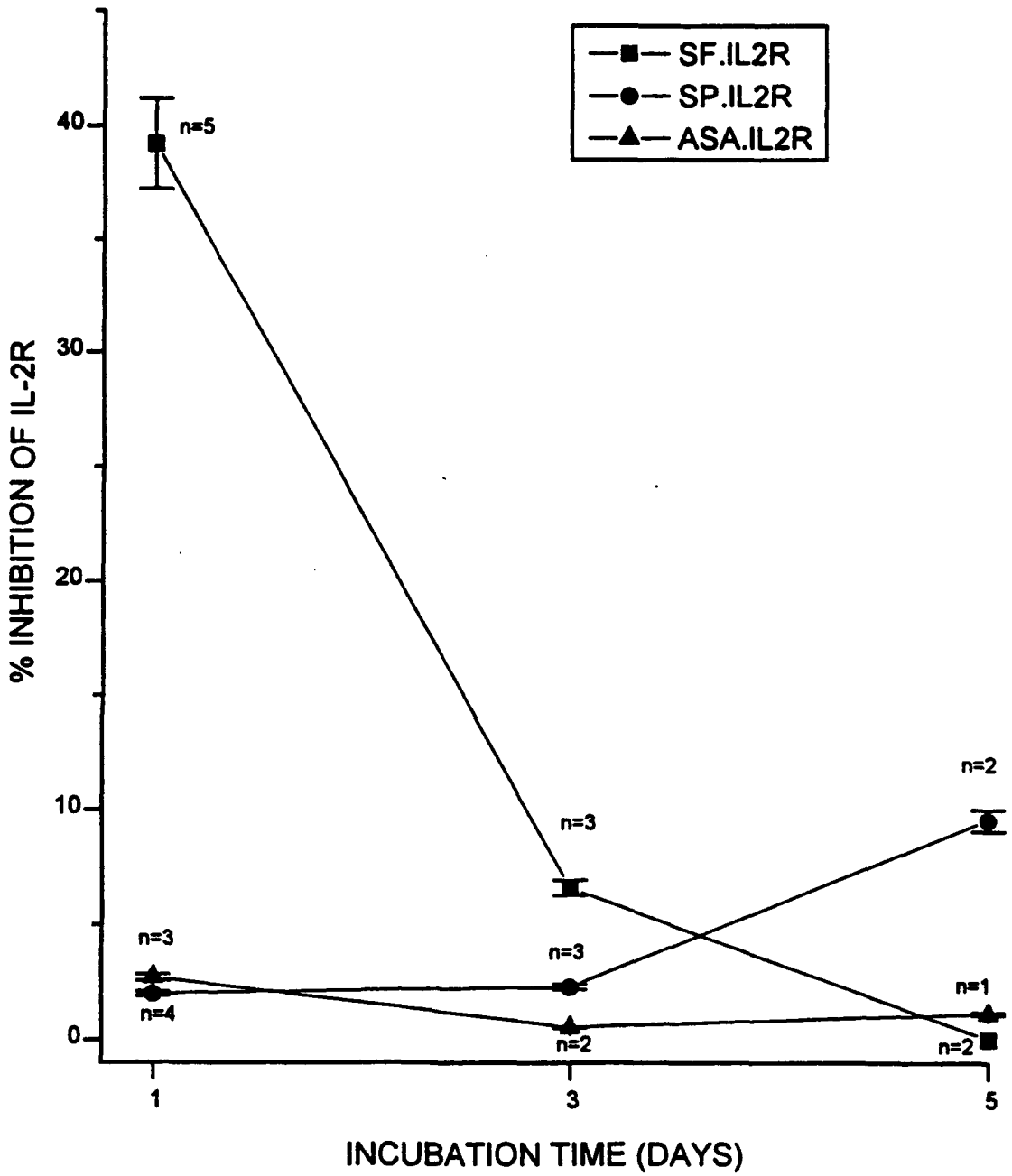


Figure 6. Effect of SF, SP and 5-ASA on IL-2R expression. Percent inhibition represents difference ([1-(X100/X0)]*100) between mean (X) percentage of T cells expressing IL-2R (CD25) in 0 ug/mL or 100 ug/mL SF, SP or 5-ASA for (n) experiments as determined by flow cytometry using FITC-mAb to IL-2R .

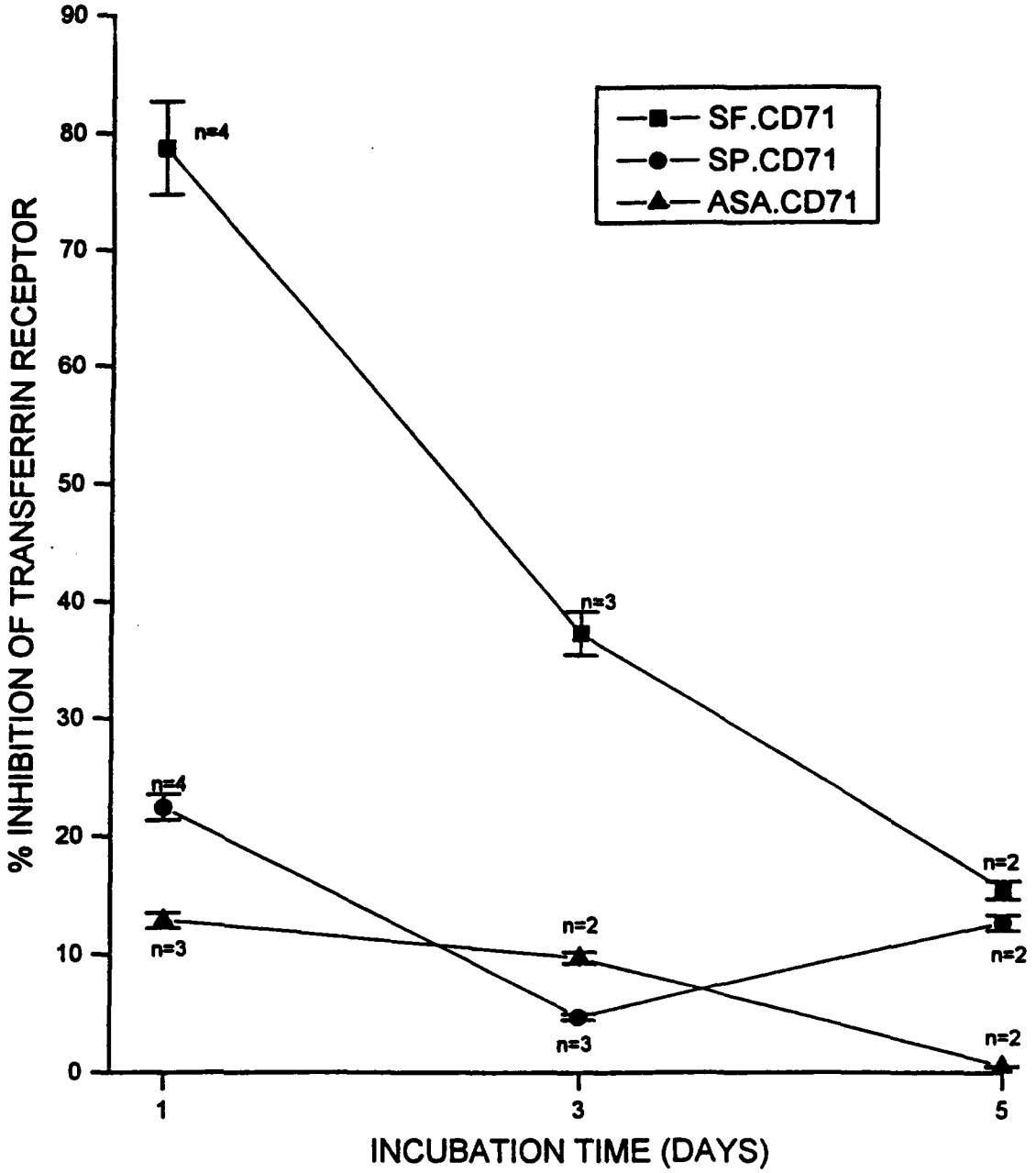


Figure 7. Effect of SF, SP and 5-ASA on transferrin receptor expression. Percent inhibition represents difference ([1-(X100/X0)]*100) between mean (X) percentage of T cells expressing transferrin receptor (CD71) in 0 ug/mL or 100 ug/mL of SF, SP or 5-ASA for (n) experiments as determined by flow cytometry using FITC-mAb to CD71.

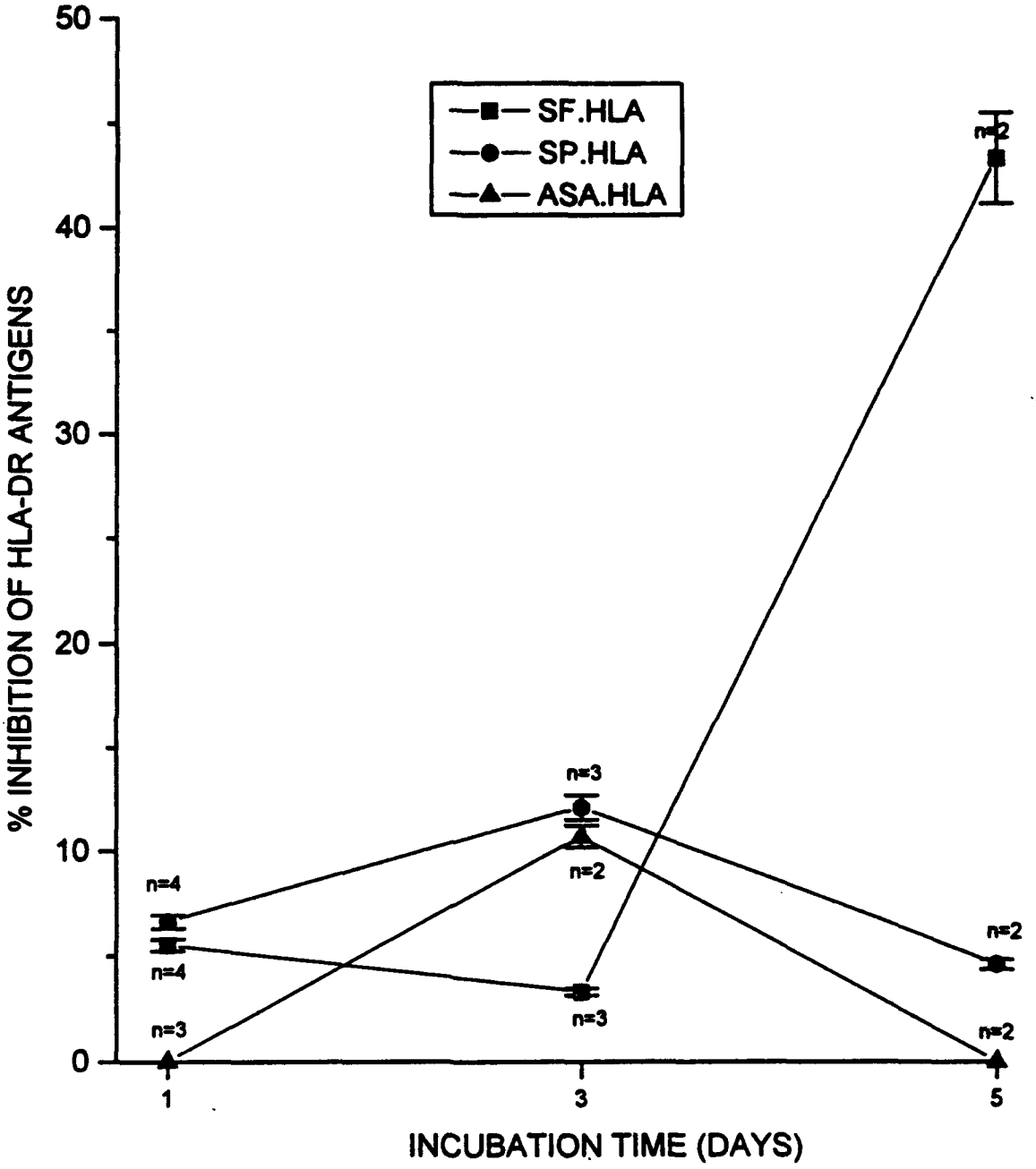


Figure 8. Effect of SF, SP and 5-ASA on HLA-DR antigen expression. Percent inhibition represents difference ([1-(X100/X0)]*100) between mean (X) percentage of T cells expressing HLA-DR antigen in 0 ug/mL and 100 ug/mL of SF or metabolites for (n) experiments as determined by flow cytometry using FITC-mAb to HLA-DR antigen.

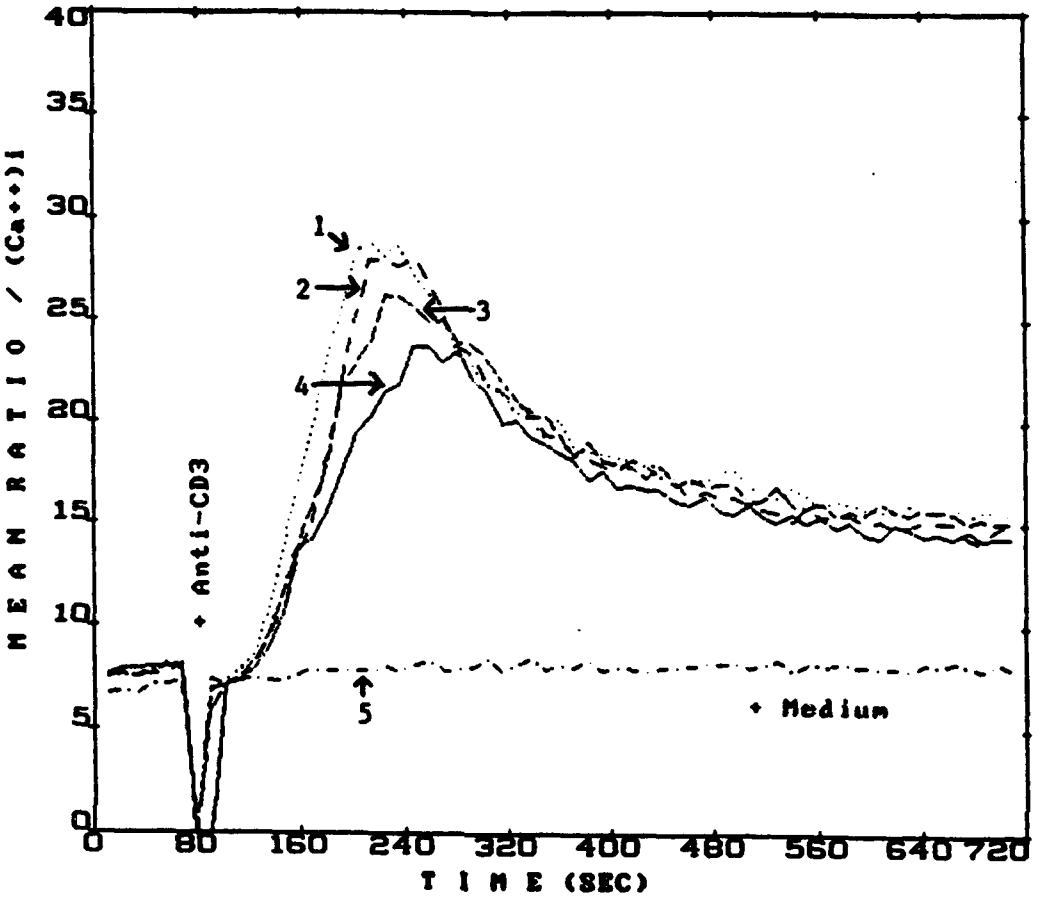


Figure 9. Mean fluorescence emission ratio of Ca^{+2}. T cells were incubated in 100 μg/mL SF, SP and 5-ASA overnight; loaded with indo-1 for 30 minutes; negatively selected by gating out with mAbs to B cells (CD19-PE) and to monocytes (CD11b-FITC) and activated with 10 μL of G19-4. Changes in ratio as a function of time were recorded for 12 minutes. The four peaks from highest to lowest ratio represent cells incubated in (1) 5-ASA, (2) SP, (3) SF, (4) G19-4 in medium and (5) medium alone (resting cell baseline Ca^{+2} ratio).

difference in the mean expression of CD71 in the two concentrations of SF on day 1 of culture was statistically significant ($p<0.05$). The expression of the transferrin receptor was mildly suppressed when cells were cultured with either SP or 5-ASA (0% to 22%), but this suppression was considerably less at all three time points than the effect of the parent drug, SF (Fig.7).

The inhibition of HLA-DR class II antigen expression in SF (Fig.8) ranged from ~3% on day 1 to 43% (34.2 ± 15.9% (SEM) mean expression at 0 μg/mL SF vs. 19.4 ± 11.1%(SEM) mean expression at 100 μg/mL SF, N=2) on day 5 of cell culture. The effect of SP and 5-ASA on HLA-DR antigen expression was constant (0%-10%) at day 1,3 and 5 after culture.

EFFECT OF SF AND ITS METABOLITES ON INTRACELLULAR [Ca^{+2}].
The effect of 100 μg/mL of SF, SP and 5-ASA on intracellular calcium mobilization was less pronounced than their effect on the three activation markers analyzed (Fig.9). The mean fluorescent ratio of the initial peak response was graphically higher and appeared earlier in cells incubated in SF, SP and 5-ASA than the mean fluorescent ratio of cells incubated in medium alone. The ratios reflecting the sustained phase (plateau region) were similar throughout the 12 min time course of the flow cytometric analysis. The G_0 cell baseline ratio was similar for all four specimens.

DISCUSSION

The present study included both analyses of mitogen-induced expression of surface activation markers, a late event in T cell activation and anti-CD3-induced analysis of intracellular calcium mobilization, considered an early event in T cell activation. The graphic results (Fig.6-9) indicate that the greatest effect on all 3 activation markers was seen with specimens incubated with SF, as opposed to SP and 5-ASA. Both metabolites appeared to elicit a milder inhibitory effect on T cell activation, if any at all. Of the three markers, CD71 was the most sensitive to drug incubation, reaching statistical significance on day 1 of culture when nearly an 80% diffference in expression in SF was measured (Fig.7). There were no discernable differences in the expression of IL-2R or CD71 on activated T cells cultured in 100 μg/mL SP or 5-ASA compared to 0 μg/mL over a five day culture period. Similar results were obtained by Comer and Jasin[15] on mitogen-induced proliferation experiments on pure B cell populations. They found a partial reduction in B cell proliferation, as well as a significant ($p<0.05$) reduction in immunoglobulin synthesis and IgM rheumatoid factor by SF, but not by its two main metabolites. Sheldon, *et al*[3] found a similar suppressive effect on mitogen-induced activation of murine spleen cells. SF caused significant suppression (>50%,

$p<0.01$) at a concentration of 50 μg/mL, but only a mild suppression (10%) was noted in SP at higher concentrations than SF.

In most experiments, although the mean expression of all three markers in SF was not significantly different compared to the control (0 μg/mL), all markers exhibited a reduced expression in the presence of SF in a time dependent manner, as opposed to SP and 5-ASA. These metabolites produced only a slight, uniform suppression, at most. This indicates that SF, but not its metabolites, may interfere with the late stages of T cell activation which involve the expression of activation markers. For CD25 and CD71, the greatest reduction in expression occurred 1 day post-incubation (Fig.6-7). For both, the percent reduction correlated well with findings of Reed, *et al*[12] that the earliest detection of mRNA for CD25 and CD71 occurred at 6 hours and 14 hours, respectively. The earlier the initial expression of the marker, the more rapid a decrease in expression occurred when SF was administered.

The graphical depiction of the percent reduction in HLA-DR class II antigen as a function of time (Fig.8) indicates a similar correlation. The largest difference in expression of the HLA antigen occurred at day five in SF (43%, n=2). This corresponded well with data from Crabtree[10] that indicated HLA-DR antigens do not exhibit a fivefold increased expression on T cells until 3 to 5 days

post-activation.

The results obtained in this study indicate that sulfasalazine, but not sulfapyridine or 5-aminosalicylic acid, may elicit an immunosuppressive effect on activated T cells as evidenced by a reduced expression of activation markers. However, more studies are required.

The effects of SF, SP and 5-ASA on calcium mobilization, which represents the early stages in T cell activation, were somewhat contradictory, compared to their effect on activation markers. All specimens exhibited a typical biphasic Ca^{+2} response (Fig.9) to the anti-CD3 mAb (G19-4) stimulus as described by Gelfand, *et al*[24]. However, the initial peak ratio caused by release of internal calcium stores was greater in the three drug cultured specimens compared to the control (no drug added). The results indicate that SF and its main metabolites may actually accelerate the initial rise in intracellular calcium released from internal cellular stores. In addition, the plateau region of the fluorescence ratio curve, attributed to extracellular calcium influx, was graphically similar for all four curves, indicating that SF and its metabolites elicited no difference in the rate of influx from the opening of calcium membrane channels. The enhanced response of the initial release of calcium may indicate an accelerative effect on the IP_3 second messenger signal pathway which involves a receptor-mediated release of

calcium from the endoplasmic reticulum. This effect did not appear to carry through to the sustained phase, indicating that SF and its metabolites does not enhance the signal pathway leading to the influx of calcium from the extracellular environment via calcium ionophores. The graphically measured threefold to fourfold increase in the 381 nm/525 nm initial peak fluorescence ratio compared to resting (G_0) T cells was somewhat less than the maximum sixfold increase measured by Rabinovitch, *et al*[20] but was still a sizable difference. The calcium results, which represent only one experiment, need to be replicated.

In conclusion, the exact mechanism of the anti-inflammatory effect of SF and its main metabolites in the treatment of rheumatoid arthritis and other autoimmune diseases is not yet known. Sulfasalazine, but not its metabolites, appeared to cause a discernable inhibition of CD25 and HLA-DR class II antigen expression, as well as, a significant ($p<0.05$) inhibition of CD71 on activated T cells, indicative of interference with late cell activation events.

A measurable positive difference in the effect of SF and its metabolites on calcium mobilization, indicated by an increase in initial peak ratios in anti-CD3 monoclonally activated T cells was observed. The results imply that SF, SP and 5-ASA may actually enhance the initial release of calcium, however, this accelerative response did not persist

completely through to the sustained phase, as evidenced by a uniform plateau region encompassing all four curves. As stated earlier, cytoplasmic calcium ion concentration ($[Ca^{+2}]$) increases, or lack thereof, are primarily associated with events that define early T cell activation.

Hopefully, the information generated from this study can be applied toward a better understanding of the possible immunomodulatory role sulfasalazine and its metabolites play in T lymphocyte activation and of the anti-inflammatory role they play in the treatment of rheumatoid arthritis and other debilitating diseases.

REFERENCES

1. Weiss A. T Lymphocyte Activation. In: Paul WE, ed. *Fundamental Immunology*. 3rd ed. New York: Raven Press Ltd., 1993:467-96.

2. Allgayer H. Sulfasalazine and 5-ASA compounds. *Gastroenterol Clin North Amk* 1992;21:643-58.

3. Sheldon P, Webb C, Grindulis K. Effect of sulphasalazine and its metabolites on mitogen induced transformation of lymphocytes-clues to its clinical action? *Br J Rheumatol* 1988;27:344-9.

4. Abbas AK, Lichtman AH, Pober JS. *Cellular and Molecular Immunology*. 2nd ed. Philadelphia:WB Saunders,1994:136-64

5. Perlmutter RM. T cell signaling. *Science* 1990;245:344.

6. Kammer G. T-lymphocyte activation. In:Rose N, Macario E, Fahey J, Freidman H, Penn G, eds. *Manual of Clinical Laboratory Immunology*. 4th ed. Washington,DC: American Society for Microbiology, 1992:207-19,231-2,877-93.

7. Weiss A. Structure and function of the T cell antigen receptor. *J Clin Invest* 1990;86:1015-22.

8. Isakov N, Scholz W, Altman A. Signal transduction and intracellular events in T lymphocyte activation. *Immunol Today* 1986;7:271-7.

9. Stryer L. *Biochemistry*. 3rd ed. New York: WH Freeman, 1988:179,459-62,975-91.

10. Crabtree GR. Contingent genetic regulatory events in T lymphocyte activation. *Science* 1989;243:355-61.

11. Leonard WJ, Kronke M, Peffer NJ, Depper JM, Greene WC. Interleukin 2 receptor gene expression in normal human T lymphocytes. *Proc Natl Acad Sci USA* 1985;82:6281-5.

12. Reed JC, Alpers JD, Nowell PC, Hoover RG. Sequential expression of protooncogenes during lectin-stimulated mitogenesis of normal human lymphocytes. *Proc Natl Acad Sci USA* 1986;83:3982-6.

13. Trowbridge IS, Omary MB. Human cell surface glycoprotein related to cell proliferation is the receptor for transferrin. *Proc Natl Acad Sci USA* 1981;78:3039-43.

14. Fu SM, Chiorazzi N, Wang CY, Montazeri G, Kunkel HG, Ko HS, Gottlieb AB. Ia-bearing T lymphocytes in man. *J Exp Med* 1978;148:1423-8.

15. Comer SS, Jasin HE. *In vitro* immunomodulatory effects of sulfasalazine and its metabolites. *J Rheumatol* 1988;15:580-6.

16. Samanta A, Webb C, Grindulis KA, Fleming J, Sheldon PJ. Sulfasalazine therapy in rheumatoid arthritis: qualitative changes in lymphocytes and correlation with clinical response. *Br J Rheumatol* 1992;31:259-63.

17. Kanerud L, Hafstrom I, Ringertz B. Effect of sulphasalazine and sulphapyridine on neutrophil superoxide production: role of cytosolic free calcium. *Ann Rheum Dis* 1990;49:296-300.

18. Sheldon P, Pell P, McBurney A. Effect of sulfasalazine on antibody response to oral antigen. *Br J Rheumatol* 1992;31:819-22.

19. Givan AL. *Flow Cytometry First Principles*. New York: Wiley-Liss, 1992:1-191.

20. Rabinovitch PS, June CH, Grossmann A, Ledbetter JA. Heterogeneity among T cells in intracellular free calcium responses after mitogen stimulation with PHA or anti-CD3. Simultaneous use of indo-1 and immunofluorescence with flow cytometry. *J Immunol* 1986;137:952-61.

21. June CH, Ledbetter JA, Rabinovitch PS, Martin PJ, Beatty PG, Hansen JA. Distinct patterns of transmembrane calcium flux and intracellular calcium mobilization after differentiation antigen cluster 2 (E rosette receptor) or 3 (T3) stimulation of human lymphocytes. *J Clin Invest* 1986;77:1224-32.

22. Finkel TH, McDuffie M, Kappler JW, Marrack P, Cambier J. Both immature and mature T cells mobilize Ca^{+2} in response to antigen receptor crosslinking. *Nature* 1987;330:179-81.

23. June CH, Rabinovitch PS, Ledbetter JA. CD5 antibodies increase intracellular ionized calcium concentration in T cells. *J Immunol* 1987;138:2782-92.

24. Gelfand EW, Cheung RK, Mills GB, Grinstein S. Uptake of extracellular Ca^{+2} and not recruitment from internal stores is essential for T lymphocyte proliferation. *Eur J Immunol* 1988;18:917-22.

25. Goldsmith MA, Weiss A. Early signal transduction by the antigen receptor without commitment to T cell activation. *Science* 1988;240:1029-31.

26. Boyum, A. Separation of leukocytes from blood and bone marrow. Isolation of mononuclear cells and granulocytes from human blood. *Scand J Clin Lab Invest* 1968;21 (Suppl 97):77-89.

27. Madsen M, Johnsen HE. A methodological study of E-rosette formation using AET-treated sheep red blood cells. *J Immunol Meth* 1979;27:61-74.

28. Walker RH, ed. *Technical Manual* 11th ed. Bethesda, Maryland: American Association of Blood Banks, 1993:690.